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LOW LEVEL LASER IRRADIATION OF NERVE CELLS *IN VITRO*

A Thesis

Presented in Partial fulfillment of the Requirements for

the Degree Master of Science in the Graduate School

of the Ohio State University

By

James Jonathan Gift, D.D.S.

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1996

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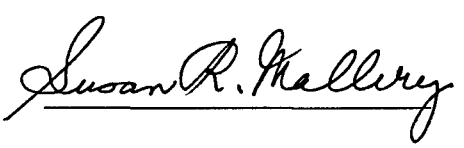

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ABSTRACT

Low energy laser treatment of patients with nerve injuries has been reported to achieve enhanced return of sensation in treated patients. Animal studies have shown reduction in scar formation and improved function following laser treatment of crushed sciatic nerves. However, these results remain controversial. Other clinical and animal studies fail to find any laser effect, and the biological basis for an effect has not been established. Studies of cultured fibroblasts have produced conflicting results, and there is little *in vitro* data regarding laser effects on nerve tissue. The purpose of this study was to determine the effects of GaAlAs low energy laser irradiation of rat cerebral cortical cells, and human nerve cells *in vitro*.

Primary rat cerebral cortical cells were obtained for the first group of three experiments. Numerous problems were encountered with growing these cells, so the protocol was modified to use established human cell lines. Human neuroblastoma, glioblastoma, and glioma cell culture lines (American Type Culture Collection, Rockville, MD) were each plated at uniform density in paired wells in multiple six-well plates. One well per plate of each cell type was then irradiated with 2, 5, or 8 Joules per day for either five or ten days, using a 70 mW GaAlAs laser system (Ronvig Instruments, Denmark). Each experimental group consisted of a total of 9 wells, each having an adjacent untreated control well. After treatment was complete cells were photographed, then harvested and

counted. A Lowry protein assay was performed on all harvested cell groups. The results were analyzed using an SPSS ANOVA analysis.

Gross microscopic observation revealed denser-appearing cultures in the irradiated groups with few exceptions. In all groups except one, mean cell counts were higher in the irradiated groups than in the paired controls. Cell counts were higher in groups treated with lower daily energy doses than in those which received higher doses (2 J > 5 J > 8 J). Protein analysis revealed a lower total protein in the irradiated versus the control groups. However, the experiments do not demonstrate statistically significant differences in cell counts or total protein between laser-treated groups and controls or among different laser-treated groups.

An *in vivo* laser effect may be the result of interactions between cell types within or adjacent to nerve tissue, or be enhanced by attenuation of laser energy by other tissues as the energy penetrates to the nerve. The present study does not support the hypothesis that there is a direct effect of the low level laser energy on perineural or neural tissue. However, the trends noted suggest that an effect might be measurable with lower levels of irradiation, as may occur at the nerve *in vivo*, and that further experimentation is warranted.

To my wife Maja, and my two boys Jamie and Wesley

for allowing me to progress in my profession,

and the love and support they gave me

throughout the past four

years, and will give

me in the

future.

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INTRODUCTION

Recovery from nerve injury such as transection or compression is often incomplete.[1-5] There are many factors which contribute to neuronal survival and axonal regeneration after injury. Axonal sprouts form within hours and grow vigorously over long distances if conditions are favorable. A variety of approaches to enhance peripheral nerve regeneration are currently being pursued. However, despite marked advancement in the understanding of the molecular and cellular mechanisms of nerve growth and repair, clinical approaches to restoration of peripheral nerve function have not changed in the past decade.

Low level lasers are becoming the forefront of medical research and treatment, however there is little understanding of the physiological effects they have on living tissues. High intensity lasers have been used as surgical adjuncts much in the same way as electrosurgery with some definite advantages. Their use is based mainly upon the absorption of light energy in the area being operated, resulting in heating of the local region and subsequent tissue destruction. The photo destruction of the tissues requires no direct contact, allowing absolute sterility. By selecting the proper wavelength, this destruction can be made selective to a certain tissue provided that the adjacent tissue differs in absorption spectrum. Ophthalmologic surgery is a good example of this

phenomenon. Depending on the focus, and wavelength of the laser utilized, surgical procedures can be performed from the eye surface to the retina. The thermal effects of the laser can also be varied to achieve: liquefaction, vaporization of surface soft tissues, surgical incision, or tissue welding instead of suture anastomosis. The putative beneficial effects of surgical lasers include: enhanced wound healing with minimal inflammatory response, decreased adjacent tissue destruction, decreased post operative discomfort, and the ability to conduct surgery with simultaneous vessel coagulation. Attention is now being turned to the therapeutic efficacy of lasers when utilized at low energy levels and differing wavelengths.[5-18]

Low energy lasers have been used clinically in Europe for more than twenty years. The energy levels produced are so low that the direct effects seen in tissues can only be attributed to the direct effects of laser radiation and not to tissue heating. Because all biomedical laser applications are based on the interaction of laser light with cellular and subcellular substances, resulting in interactions that can cause a broad spectrum of effects. Low level laser light can be absorbed, reflected, or reradiated by a substance or tissue so that no changes occur within it. This is the basis for diagnostic laser applications. Laser energy may produce excited electronic states in biological molecules, leading to a photochemical transformation at the molecular level.[19]

Fundamental to the applications of lasers are their unique properties. Lasers have spacial coherence and polarization, allowing the beam to be focused in minute diameters. The laser beam is monochromatic allowing excitation of certain molecules and absorption or transmittance within tissues. Lasers are tunable in a range from ultraviolet to infrared,

however the most common wavelengths used for therapeutic purposes is in a wavelength range between 630 nm and 1300 nm, visible to infrared. The parameters which describe low level lasers are type, wavelength, average power, time, area of irradiation, and beam mode, which is either continuous or pulsed. The incident dose of the laser is measured as either power density, or energy density. Power density is defined as output power in watts divided by area of irradiation, (mW/cm^2). The energy density is defined as output power times irradiation time, divided by the area of irradiation, (J/cm^2). Energy levels for low level lasers are in milliwatts while surgical lasers are in watts.[6,19,20]

Research utilizing low level lasers can be divided into three basic areas: animal experiments, human trials, and cellular function. To date the reported observations and treatment outcomes are not fully understood and there is contradictory evidence in the current literature regarding the effects of low level lasers. The focus of most reports has been on observations of animal experiments and human trials, with little emphasis on the cellular level. It is the purpose of this project to determine the effects of a low level GaAlAs laser on nerve cells *in vitro* with the hypothesis that lasers stimulate growth of neural and perineural cells.

REVIEW OF LITERATURE

Nerve Regeneration Following Injury

The pathophysiology of nerve regeneration following injury is a complex process that depends on the interaction of the nerve cell body, the axon, and the surrounding cellular environment.[1-5] The initial response following injury is leukocyte activation and macrophage chemotaxis to the region of trauma. Next, there is destruction of the remnant structures in the distal axon with phagocytosis of debris. Also, calcium ions enter into the axoplasm, subsequently activating various proteases and apoptosis associated enzymes resulting in tissue and cellular destruction. Notably, axonal transport is maintained throughout this repair/regeneration process.

The distal axonal segment degenerates slowly in a process termed wallerian degeneration.[1-5] During this process the myelin sheath retracts from the axon and degenerates. These endoneurial tubes then serve as conduits that guide regenerating axons to their targets. However, the Schwann cell body remains intact and secretes extracellular proteins that promote axonal extension.

Chromatolysis, which entails nuclear swelling and peripheral margination of the rough endoplasmic reticulum, occurs within the nerve cell body.[1-5] This marginated

endoplasmic reticulum is known as Nissl substance. Chromatolysis, a component of apoptosis, is associated with increased RNA and protein synthesis.

In addition to phagocytosis, macrophages perform other vital functions in neural regeneration.[1-5] Macrophages secrete numerous mitogens and growth factors inclusive of: nerve growth factor (NGF), insulin-like growth factor (IGF), platelet derived growth factor (PDGF), and apolipoprotein-E, as well as substances that aid in Schwann cell proliferation. The concentration and function of these numerous trophic factors modulate and influence neural survival.

During regeneration the axon sends multiple sprouts distally.[1-5] At the distal sprout tip is a specialized structure known as the growth cone, which is comprised of neurites that extend from the proximal stump and search for a supportive environment to maintain axonal growth and regeneration, i.e. Schwann cell tubules. It is speculated that this process is mediated by neurotropism-chemotaxis and contact guidance.

Regeneration depends on the survival of a sufficient number of neurons.[1-5] Gaps between proximal and distal axon stumps needs to be bridged successfully. Sufficient axonal growth must not only occur, but the axon must make the correct connection with its end organ. A variety of approaches to enhance nerve regeneration are being pursued including irradiation with low level lasers.

Animal Experiments

Effects of low level lasers on wound healing in animal models has been extensively investigated.[21-37] Much of the early literature has been from the European countries, and

has not been translated into English. In 1971, Mester and associates reported on the effects of a ruby laser on wound healing in rats.[21] From their previous studies they reported that low energy lasers stimulated phagocytosis, fur regeneration, neoplastic growth, and healing of mechanically induced wounds. In their 1971 study, they used a ruby laser with doses of 1.0 J, 4.0 J, and 5.0 J on bilateral third degree burns in white mice. It was determined that wound healing was accelerated on the irradiated side versus the control side in eight of ten mice with exposure to low level laser irradiation. They concluded that laser radiation stimulates wound healing by stimulating epithelial growth. However, their sample was small, consisting of ten mice.

Dyson and Young evaluated the effects of low energy HeNe lasers on wound contractility and cellularity in the mouse model.[22] Their experiments involved surgical wounding in three groups of six animals. One group was irradiated with a 700 Hz laser, one group irradiated with a 1200 Hz laser, and the third group served as the control. They reported different observed effects between the two experimental groups, as well as the control group. There was an observed increase in wound contraction at the lower frequency, and an inhibited contraction in higher frequency radiation groups, however there was no significant difference when the groups were compared with the non-irradiated control. When the two irradiated experimental groups were compared against each other, there was a significant difference seen in wound contracture in the first two weeks of healing. They also noted an increase in the fibroblast count of the two experimental groups versus the control. The lower energy group had the highest

fibroblast count and the control the lowest count, however there was no statistically significant difference reported.

Rochkind et al. has published a series of articles on the effects of the HeNe low level laser on induced nerve injuries in rats.[23-26] In their earlier studies it was reported that: 1) action potentials increased in irradiated sciatic nerves in normal rats[23], 2) there is an increase in the action potential in irradiated cut sciatic nerves versus non-irradiated controls, and that these effects are long lasting.[24] Under 3J of energy, there was no change in the action potential, and once the energy exceeded 9J, that an inhibitory effect was appreciated. Rochkind et al. also reported that morphologically laser irradiated injured nerves exhibited less scar tissue as compared to control wounds.[24-26] Later studies showed enhanced bilateral recovery in cutaneous wounds, burns, and nerve crush injuries when the low energy laser was used on only one side as compared to control groups.[25] At this time they also indicated that these effects diminished as the wavelength decreased from 632 nm to 465 nm. Rochkind et al. stated that these findings reflected enhanced and accelerated functional and morphological recovery of severely injured tissues. They also reemphasized that the observed effects were long lasting. More recent work by Rochkind et al. showed an increase in the number of glial cells in intact irradiated nerves, injured nerves, and irradiated injured nerves compared to non irradiated control rats.[26] In this study they also observed less degeneration of the Nissl bodies and postulated that protein synthesis was increased in the irradiated nerves. However, there was no protein analysis or statistical analysis performed on this study.

Other studies have reported negative findings regarding the benefits of lasers on wound healing.[27-29] Khullar and colleagues studied the effects of an 830 nm GaAlAs low level lasers on the recovery of nerve conduction and motor function after crush injury in rat sciatic nerves.[27] Although irradiated animals had improved functional recovery after 21 days, the investigators did not notice a difference in evoked potentials or histologic changes between the irradiated and non-irradiated groups. It was concluded that the effects were most likely peripheral to the point measured. Anneroth et al. used a 904 nm GaAs laser on induced wounds in the rat model.[28] Notably, the energy delivered to the tissues in J was not indicated. However, no differences were found in comparison of wound healing between irradiated and non-irradiated control wounds at the gross or histologic levels on the same animal. There was also no morphologic differences seen in the two groups of wounds. These investigators did not detect improvement in wound healing using a low level laser. Smith et al. could also not confirm beneficial effect of the HeNe laser on skin flap healing in the rat or porcine models.[29]

Other investigators have assessed the gross and cellular effects of lasers on surgically induced and sutured wounds.[30-32] Lyons et al. reported that visual examination revealed no difference in HeNe laser treated incisions in mice compared to incisions in control animals.[30] However, there was an increased tensile strength in the irradiated wounds, but it was not statistically significant. Biochemically, there was a significant increase in collagen deposition in the laser treated wounds at 2 and 4 weeks. Takeda found fibroblast proliferation and increased osteoid formation in GaAs laser treated tooth extraction wounds in rats.[31] Increased lead deposition in the newly formed bone also

suggested more rapid ossification in the irradiated group. Yew et al. found increased PAS-positive material between pigmented epithelium and the visual cells following HeNe laser treatment in albino mice retinas.[32] Quantitative analysis of ^{3}H-labeled uridine revealed a statistically significant increased uptake at 4 hours post treatment in irradiated animals. A second uridine labeling at 24 hours showed no significant increase between the two groups. This increased uptake was postulated to indicate an increase of RNA production within the irradiated cells.

Interest has also been generated regarding laser effects upon components of the CNS. Lavie and colleagues studied optic axons in rabbits and determined that axonal growth occurred distal to injury in optic nerves irradiated with HeNe low level lasers, while no distal axonal growth occurred in control animals.[33] These investigators have also implanted fish optic nerves into injured rabbit optic nerves and noticed growth enhancement when the site is irradiated with the HeNe laser.[34]

Analgesic effects of low level lasers have been both reported and refuted in animal models.[35-37] Mezawa et al. observed reduced firing rates in GaAlAs irradiated cat tongues.[35] This effect peaked with a ten minute treatment. This suggests analgesic effects, and confirms increased evoked potentials as seen in other animal models. Zarkovic et al. however indicated shortening of latency, thus stimulation of pain in mice irradiated with a GaAs laser.[36] Jarvis et al. also concluded that HeNe lasers had no effect on peripheral A*d* or C-fiber nociceptors in the rabbit cornea.[37] These conflicting results are apparent in human trials as well.

Human Trials

Low level lasers have been in use for over twenty years to treat a variety of human diseases. However, much of the human treatment with lasers has been on an experimental basis. Most of the reports do not attempt to elucidate the mechanism of action of the therapeutic laser. The main use for low level lasers is in physical therapy where they are used to treat arthritic conditions, decrease pain, and promote healing of soft tissue injuries both immediate and delayed.[38] The major problem is determining the optimal treatment parameters of these devices. But, patients readily accept laser treatment due to its painless and high tech nature. It has been reported that patients also have higher expectations from laser treatment than conventional means of physical therapy, because they believe it to be state of the art.

Walker and Akhanjee observed an apparent photosensitivity of peripheral nerves.[39] Their results indicated that HeNe lasers stimulate low threshold myelinated fibers similar to effects observed with electrical stimulation. However, prolonged exposure to the laser attenuates the subsequent response to electrical stimulation. This was seen with the decrease in amplitude of the evoked potential of the laser stimulated nerves, which increased with continued laser exposure. The electrically stimulated nerves exhibited no amplitude change with continued stimulation. Wu et al. repeated the experiments but were unable to verify the claims made by the previous investigators.[40] Further trials were conducted on median nerves in normal volunteers.[41-43] Bashford's group found a small but not statistically significant decrease in motor and sensory distal latency in irradiated subjects using a 830 nm GaAlAs laser.[41] But, they found no differences in action

potentials or skin temperatures between treatment and control groups. Their conclusion was that lasers can affect median nerve function, however its effects are limited. This was also confirmed by Baxter and his coworkers using a similar laser.[42,43]

Pain relief has also been investigated. Analgesia was observed in 9 of 26 patients receiving low level radiation over the medial, radial, saphenous, and involved painful nerves.[44] Notably, patients receiving sham treatment reported no analgesia. Walker also observed large increases in urinary excretion of 5-hydroxyindoleacetic acid, the degradation product of serotonin in the irradiated patients only. Carillo and his colleagues compared the effectiveness of HeNe laser, ibuprofen, and placebo on pain prevention and swelling after third molar removal.[45] They reported less trismus in the laser and ibuprofen groups, but that post operative pain was significantly less in the ibuprofen group compared to the laser and placebo groups. There was also no difference in swelling among the three groups. Lim et al. used a GaAlAs low level laser to treat post orthodontic manipulation pain.[46] Patients exposed to the laser reported a lower level of pain compared to the placebo treated group, although statistical analysis of the data failed to show a significant difference.

There has also been interest in recovery from nerve injury using low level lasers. In a treatment only study by Haanaes, patients who sustained injury to the inferior alveolar, mental, or lingual nerves after dental procedures were analyzed.[47,48] The patients underwent laser treatments using a GaAlAs laser at a wavelength of 830 nm. The treatment was directed over the area of the involved nerve closest to the tissue surfaces, i.e. mental foramen, lower lip and chin for mental nerve injuries; internal oblique ridge and

lateral border of tongue for lingual nerve injuries; and mandibular foramen, mental foramen, third molar area and lower lip for inferior alveolar nerve injuries. A total of 6 J was administered per site at each treatment. Improvement of 40-90% was noted in the eight patients with clinical symptoms present for less than 1 year after ten treatments. Three of these patients continued to 20 treatments, and noted an improvement of 60-80%. The remaining 32 patients had residual long term symptoms. This group reported an improvement of 40-80% and 60-90% after ten and twenty laser treatments respectively. Poole et al. used a similar protocol in a blinded controlled clinical trial at Ohio State University using the same type laser.[49] Fifteen total patients were evaluated, 8 in the treatment group, and 7 in the control group. Neurosensory testing consisting of static two point discrimination, moving two point discrimination, and VonFrey fiber testing which were performed pre treatment, and after ten treatments. Treatments were administered as in the previous study with 6 J of energy per site. There was a subjective improvement seen in both treated and nontreated groups. While there was no significant difference between the two groups with the static and moving two point discrimination tests, there was a significant improvement in the upper half of the mental region in the treatment group compared to the sham irradiated control group. These two clinical human studies suggest that low level lasers exhibit some therapeutic and potentially regenerative effect on damaged nerves.

Cellular Function

Few *in vitro* studies have been conducted to clarify the cellular effects of low energy lasers.[50-52] Experiments performed by van Breugel et al. have shown that a 630 nm HeNe laser stimulates cell proliferation of human fibroblast in culture, while concurrently decreasing cellular production of type I collagen.[50] This study also evaluated the effects of variable power settings and exposure times and concluded that lower power settings and shorter exposure times had the greatest effects on fibroblast proliferation as determined by cell counts. These findings suggest a dual cellular effect-decreased collagen production and increased cellular proliferation. This study did not compare cellular morphology between the irradiated groups nor was there a non irradiated control group. Other studies by van Breugel on rat schwann cells indicated that the wavelength of the laser is important as well.[51] Lasers with wavelengths of 670 nm and 830 nm showed an increased cell proliferation of 40% and 30% respectively compared to non-irradiated controls. Lasers with a wavelength of 780 nm had inhibitory effects of 30% on the same cells compared to the controls. These findings probably reflect the fact that the wavelength of laser light may be responsible for activation different processes within cells. The laser type was not indicated in this study. Yu et al. used a 660 nm argon dye laser on 3T3 fibroblast cultures and noted a statistically significant proliferation of cells that were irradiated at 2 J versus cells irradiated at 3.5 J as compared to non-irradiated control cells.[52] He also showed that this effect could be inhibited by addition of basic fibroblast growth factor (bFGF) antibody. This suggests that laser irradiation may involve increased production of bFGF in fibroblasts.

Despite the extensive clinical and animal experimentation conducted on laser exposure and nerve function, only several investigators have done in vitro studies.[53-54] In 1971 Fork discovered during his nerve mapping experiments that laser radiation at 488 nm selectively stimulated neurons in the abdominal ganglion of *Aplysia californica*, a marine mollusk.[53] While lasers with wavelengths in the near infrared regions had no effect. He also noted that there was no observable damage to the neurons. Olson' work concentrated on rat cerebral cortical cells *in vitro*.[54] Using six different wavelengths from 490 to 685 nm and an energy flux from 0 to 100 uJ he stated that laser pulses above a threshold significantly reduced the cells' excitability as measured by electrical stimulation. He postulated that these results were due to laser light absorption in the mitochondria with subsequent release of calcium, but he did not indicate the threshold energy. If these observations made in animal experiments, and human trials, and the results of these fibroblast studies can be extrapolated to nerve cell cultures, then one would anticipate that laser irradiation will have a stimulatory effect on neural proliferation.

MATERIALS AND METHODS

Rat Cerebral Cortical Cell Isolation and Culture

Primary rat cerebral cortical cell cultures were established using methods described by Jordan and Thomas[55-58], with modifications by Brewer et al.[60,61] A 15 to 18 day gestation timed-pregnant Sprague-Dawley rat was sacrificed via cervical dislocation, the uterus immediately removed under sterile conditions, and placed in a dissociation medium consisting of: calcium and magnesium free Hanks' balanced salt solution, supplemented with 1M sodium pyruvate, and 10 mM HEPES, at pH 7.4. All embryos were then dissected from the uterus and medial occipital tissue removed. After transfer to fresh dissociation medium, the tissue was disrupted by trituration ten times using a sterile pipet. Divalent cations were restored by dilution with 2 volumes of Hanks balanced salt solution supplemented as above. Non-dispersed tissue was allowed to settle for 3 minutes and the supernate was then transferred to a 15 ml tube and centrifuged at 200g for 2 minutes at 4^0C. The supernate was discarded and the pellet was resuspended in 1ml Hanks balanced salt solution per brain. An aliquot was taken for cell enumeration and viability (trypan blue exclusion). The cells were plated at a minimum of 5×10^4 cells per dish.

Culture dishes were prepared in three different ways for the first experiment. Sterile six well tissue culture dishes, (well diameter 35 mm) were used for the majority of the experimental groups. One group of culture plates had a 1/4" hole drilled in the bottom and a 22mm diameter round glass coverslip (Fisher Scientific Cat. #12-545-101) was attached to the outside bottom of the dish using paraffin tissue preparation embedding media. These dishes were then UV irradiated in a laminar flow sterile hood for 10 minutes. The second set of culture plates had a autoclave sterilized coverslip placed in the bottom of each well. These cover slips were not attached. The third group were used as supplied from the manufacturer with no modification. All subsequent experiments using primary cell cultures used unmodified (i.e. no coverslip) culture plates. The culture plates were coated with 3ml of a solution of 0.05mg/ml cold poly-D-lysine (MW 30,000-70,000) or poly-L-lysine (MW >300,000), and allowed to incubate overnight at 37^0C. The dishes were washed with sterile water and allowed to dry 1 hour before use. Two ml of tissue culture media was placed in each well prior to tissue dissection and stored in a 37^0C incubator with 5% CO_2. The initial culture media was Neurobasal (GIBCO BRL Cat.# 211103-023) supplemented with 0.5 mM L-glutamine, 25uM glutamate, B27 50x supplement (GIBCO BRL Cat.# 17504) and 5% heat inactivated fetal bovine serum. This medium was used for initial plating only. The media was changed to B27 supplemented Neurobasal at day 4. This serum free media was used for all subsequent media changes.

The dishes were divided into ten equal groups, each consisting of 1 six well plate. Two groups served as controls. The remaining groups were irradiated with a 70 mW GaAlAs laser diode, wavelength 820-830 nm continuous, nonpulsing from Ronvig

Instruments, Denmark. Two groups were irradiated at 2 J, one daily and the other every other day, two groups at 4 J , two at 6 J, and two at 8 J as above starting at day 2 after plating. Observations were made on a daily basis using a phase contrast microscope at 100x magnification. Cells were observed by one investigator and notations recorded regarding growth characteristics, amount of cellular debris produced, and rate of growth to confluence.

In the initial experiment one of the control dishes was characterized at day 7. The immunoassay was performed using a dual staining technique. This consisted of primary staining with neuron specific enolase and glial fibrillary acidic protein (GFAP) and secondary staining with rhodamine anti neuron specific enolase and fluorescein anti GFAP. The cells were then observed under florescence microscopy using the appropriate wavelength parameters. Confirmation of cerebral cortical cells was verified.

Three experiments were conducted using primary cell cultures. In the first experiment plating density was 1.54×10^5 cells per well, or 160 cells per mm^2. In the second and third experiments, cells were plated at 2.25×10^5 cells per well, or 234 cells per mm^2; and 3.5×10^5 cells per well, or 200 cells per mm^2, respectively. The third experiment used standard 24 well tissue culture plates instead of 6 well plates, however, due to contamination with Aspergillus species, this experiment was terminated.

Cell lines

Isolation and culture of racine primary cell cultures proved to be time consuming, prone to contamination, and difficult to grow reliably. Therefore, human nerve cell tumor

lines were obtained from the American Type Culture Collection (ATCC), Rockville, MD to continue this pilot study. The cell lines were chosen for their growth characteristics and human neural/perineural origin. Three cell lines were obtained: human neuroblastoma, (HTB-11), human glioblastoma, (HTB-16), and human glioma, (HTB-138).

ATCC HTB-11 is a human neuroblastoma (neural crest origin) obtained from a 4 year old Caucasian female. It was developed by J. L. Biedler in 1970 and has been used extensively in cell mediated cytotoxicity assays. It is described as the SK-N-SH line. HTB-11 has an epithelial like morphology, and grows as a monolayer . It transfers 1:12.5 weekly, and is not tumorigenic. In vitro cytopathology is typical neuroblastoma. Culture medium is antibiotic-free Eagle's minimal essential medium (MEM) supplemented with non-essential amino acids, sodium pyruvate, and Earle's balanced salt solution (BSS), 90%; and fetal bovine serum (FBS), 10%.

ATCC HTB-16 is a human glioblastoma obtained from a 47 year old Caucasian male. It was developed by J. Ponten in 1971. It is described as the U-138 MG line. HTB-16 has a polygonal morphology, and grows in a monolayer. It transfers 1:3.5 weekly, and is not tumorigenic. *In vitro* its appearance is spindle shaped cells consistent with glioblastoma. Culture medium is antibiotic-free Eagle's minimal essential medium (MEM) with non-essential amino acids, sodium pyruvate, and Earle's balanced salt solution (BSS), 90%; and fetal bovine serum (FBS), 10%.

ATCC HTB-138 is a human glioma obtained from a 76 year old Caucasian male. It was developed by the National Cancer Institute in 1976, and is described as the Hs 683

line. It has a fibroblast like morphology, grows in a monolayer, is transferred 1:4 weekly, and is not tumorigenic. *In vitro* cytopathology is consistent with glioma. Culture medium is antibiotic free Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 90%; and FBS, 10%.

The cells were grown in their optimal media. HTB-11 and HTB-16 were grown in Eagle minimal essential medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and Earle's BSS, 95%; and 5% FBS. HTB-138 was grown in Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids, 95%; and FBS 5%. At least 48 hours prior to use, representative media aliquots were incubated (37^0C, 5%CO_2) to rule out microbial contamination.

Cell Expansion

Upon arrival the cells were thawed and placed in sterile 15 ml conical centrifuge tubes. All cell manipulations were performed under a sterile laminar flow hood. The tubes were centrifuged at 4°C, and 1500g for 4 minutes. The supernate was discarded and the pellet size assessed. The pellet was then resuspended in 5 ml of the appropriate media, and transferred to a sterile T-75 filtered flask containing 15 ml of media. The cells were grown for 7 days at 37°C with 5%CO_2. Half of the media was changed at day 4. All flasks exterior surfaces were cleaned with 70% ethanol or sodium hypochlorite prior to being placed under the hood. At 7 days the cells were harvested. The media was decanted and the cells washed 3 times with phosphate buffered saline solution (PBS). The cells were

then trypsinized using 1 ml of 0.05% trypsin/EDTA per T-75. The flasks were placed in the incubator for 5 minutes. This proved to be the optimal time to allow the cells to release from the flasks. The trypsin was then deactivated by adding 4 ml of media and the flask was gently agitated. The resuspended cells were then transferred to a 15 ml conical centrifuge tube. The tube was centrifuged for 5 minutes at 1000 rpm, at 4°C. The supernate was then discarded and the cells resuspended in 4 ml of media. Half of the suspension was then used for continued expansion , 1ml per T-75, and the other half was used for freezing. An aliquot was taken for cell counts and viability testing using trypan blue using a standard hemocytometer.

Cell freezing

The suspension remaining from cell line expansion was centrifuged for 5 min at 2000 rpm and the supernate discarded. The pellet was resuspended in 2 ml of 90% media, 10% DMSO. The DMSO/media cryopreservation storage medium was sterile (0.2 uM) filtered prior to use. The new suspension was divided into 1 ml aliquots and placed in labeled cryovials. The cryovials were then placed in an alcohol freezing container and placed in a -80°C freezer over night, minimum 8 hours. The following day the cryovials were transferred to liquid nitrogen storage, for later use.

Experimental design

When a sufficient numbers of cells had been obtained through expansion and freezing techniques, the cells were plated into six well sterile tissue culture plates in

optimal media. The HTB-11 cell line was plated into the top two wells of each plate, HTB-16 cell line in the middle two wells, and the HTB-138 cell line in the bottom two wells, and the plates were labeled as shown in figure 1.

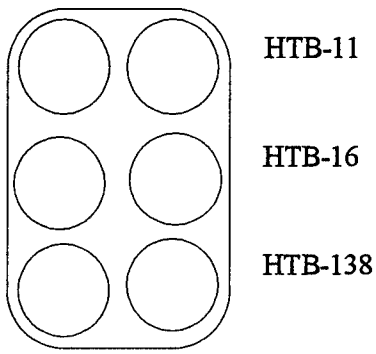


Figure 1:Culture plate cell arrangement

A total of eighteen plates were used for each experimental trial. The experiment was repeated three times. The plates were separated into three groups containing six plates each. Each tissue culture plate was then subdivided into right and left sides. The left half of each plate was irradiated using a low level GaAlAs laser diode, and the right side served as the control. Each of the three groups would receive a different energy level of irradiation using the GaAlAs laser diode. One group would receive 2 Joules of irradiation, one group would receive 5 Joules, and the third group would receive 8 Joules, as shown in figure 2. Additional doses were delivered at a minimum of eight hour

intervals to allow for cell recovery. The irradiation was performed through the top of the tissue culture dish on the bench top in the tissue culture lab. After five treatments, three dishes were randomly selected from each group for photomicrographs; cell harvesting, viability assessment and counting, and total protein analysis using a Lowry assay.

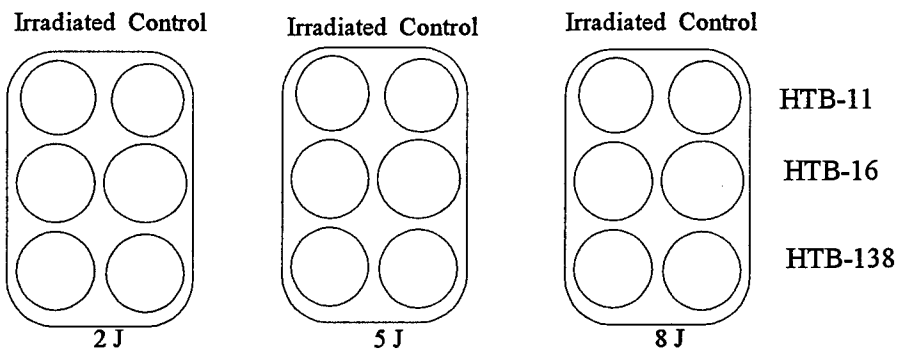


Figure 2: Culture plate experimental setup

GaAlAs Laser Diode

The GaAlAs laser diode is a class 3 B semiconductor based laser system for low level laser therapy. It is called the Photon Plus and is manufactured by Ronvig

Instruments, Denmark. The laser source is a GaAlAs semiconductor which has a power of 70 mW maximum using the straight light guide. Effective beam transmission is 95%. This was confirmed by the bioengineering department at the Ohio State University Medical Center. The wavelength is 820-830 nm in the near infra-red region and the energy delivered is continuous non pulsating. The beam diameter is 0.45 cm with an area of 0.16 cm^2. Beam divergence is < 1% due to the collimating lens. The total energy dosage is a function of the measured power in mW, measured internally when the laser is turned on, and the total irradiation time. Energy in Joules (J) = Power (mW) x time (sec). Power in mW is established by the laser when it is switched on, and the joules or the time can be set independently, with the calculation of the third variable.

Radiation Schedule

The culture plates were plated as previously described and seen in figure 1. The cells were allowed a minimum of 8 hours to adhere to the culture plates before the initial irradiation. The left side of each 6 well plate was irradiated, and the right side served as a non-irradiated control. One group of six plates was irradiated at 8J, one group of six plates irradiated at 5 J, and the third group of six plates irradiated at 2 J. A minimum of 8 hours was allowed between subsequent irradiation dosages to allow for cell recovery. After 5 treatments, three plates from each group were randomly selected for photomicrographs, cell harvesting and counting, and protein analysis. The remaining three plates in each group were irradiated five more times, for a total of 10 treatments. Photomicrographs were then taken on these plates, as well as, cell harvesting, counting,

and protein analysis. Each individual well was harvested and counted separately. A protein analysis was also performed on the counted cells from each well. There was no pooling of samples.

Microscopic Observation

The cells were observed under phase contrast microscopy prior to each irradiation cycle to assess the presence or absence of morphologic differences, increased mitotic activity, cell density, number and confluence between the irradiated and non irradiated controls. After completion of five treatments, three plates were selected from each irradiation group and photomicrographs were taken. This process was repeated for the remaining three plates in each group after the tenth treatment. Photographs recorded the cellular morphology and growth characteristics of the irradiated and non irradiated control groups permitting comparison between the three repeated experiments. A representative well from each cell line and irradiation group were photographed using a Nikon inverted diaphot-tmd microscope. The irradiated well, and the adjacent control well were both photographed. Image scale was 25x and 50x (2.5x internal and 10x & 20x objective) using the appropriate phase contrast for each magnification. Thirty five mm slides using Fujicrome Sensia ASA 100; and black and white prints using Kodak T MAX ASA 100 were taken of each well. After taking photomicrographs, the plates were prepared for cell harvesting.

Cell Harvest

The plates were disinfected and placed in a cell culture hood. The media was aspirated, the wells were rinsed three times with PBS, this was discarded, and the wells were then trypsinized using 0.2 ml trypsin/EDTA per well. The plates were incubated for three minutes at 37^0 C. The plates were removed from the incubator, disinfected, and gently agitated to ensure release of the cells from the culture dish. Then, 1.2 ml of media was added to each well to deactivate the trypsin. The plates were gently agitated again, and the suspension was transferred to labeled microfuge tubes using individual sterile pipettes, and centrifuged at 14,000 rpm at 4°C for 5 minutes. The supernate was discarded, and the pellet resuspended in 1.0ml PBS. The microfuge tubes were refrigerated prior to assessing cell viability and numbers.

Cell Count and Viability

Cell counts and viability were determined using a standard hemocytometer chamber as illustrated in figure 3. A micropipette was used to mix 10ul of a 1:2 dilution (10ul of the thoroughly agitated cell suspension to 10 ul 0.4% Trypan Blue) was prepared. The mixture was allowed to stand for 5 minutes prior to counting, then transferred to the hemocytometer with the coverslip in place using a micropipette. The chamber was allowed to fill by capillary action. Cells were counted in the center square, and each corner square of the hemocytometer, keeping separate totals for nonviable and total cells. A viable cell demonstrated trypan blue exclusion. Cells were counted if they were in contact with the top and left lines for each 1mm square, and not counted if they

were touching the right or bottom lines, as indicated in figure 4. Cell counts were determined by the following calculations:

1) CELLS PER ml = average count per square x dilution factor (2) x 10^4

2) TOTAL CELLS = cells per ml x the original volume from which the sample was removed

3) CELL VIABILITY(%) = total viable cells (unstained) / total cells x 100.

This procedure was performed for each well, no samples were pooled.

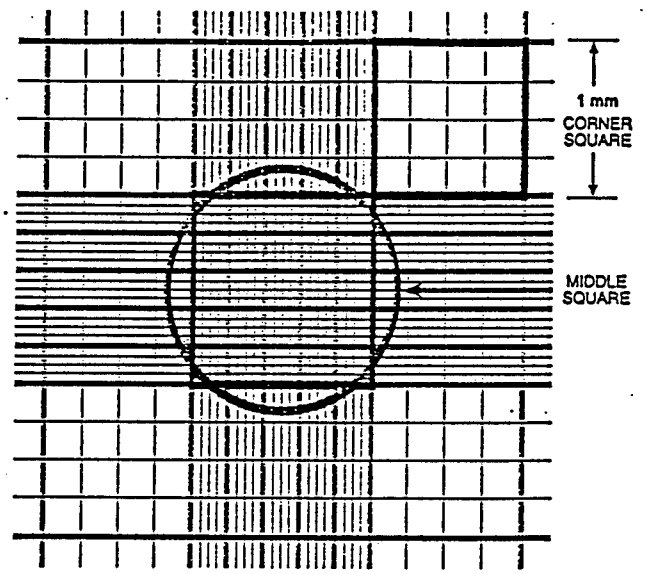


Figure 3: Standard hemocytometer chamber. The circle indicates the approximate area covered at 100x microscope magnification (10x ocular & 10x objective).

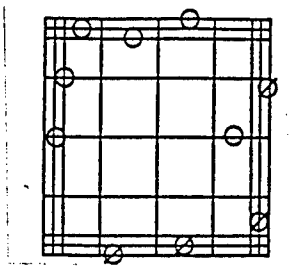


Figure 4: Corner square enlargement. Count cells on top and left touching middle line. Do not count cells touching middle line at bottom and right.

Lowry Protein Assay

After completion of cell counts, the microfuge tubes with the remaining cells were centrifuged at 14,000 rpm at 4°C for five minutes. The tubes were checked for pellet prior to aspirating the supernate. The pellet was resuspended in 0.5ml PBS to which 0.5ml 2M $HClO_4$ was added. The protein precipitated from the suspension, and the samples were stored in -20°C until the day of the assay. On the day of the Lowry protein assay[62], reagents for the assay were prepared from fresh stock solutions. Reagent D: 75ml 2% Na_2CO_3, 750ul 1% $CuSO_4$, and 750ul 2% NaK Tartrate. Reagent E: 20 ml folin reagent, and 180 ml 0.1 N NaOH. The samples and standards were removed from the freezer and thawed by warming in hands. Once thawed, the samples were centrifuged at 14,000 rpm for 3 minutes. The supernate was pipetted from the pelleted standards and the cell samples with care not to disturb the pellet. If the pellet was disturbed, the sample was recentrifuged. One hundred ul of 1M NaOH was added to the microfuge tube. It was then vortexed until the pellet dissolved, placed into a styrofoam holder, and incubated

in a water bath set at 50°C for 30 minutes. Since the assays contained 64 total protein samples, the standards were run first, and the rest of the samples put in groups of 18 and preparation was staggered to optimize incubation and development times. After the incubation time, the samples were removed from the water bath and 1.0 ml of reagent D added. They were vortexed, and transferred to previously labeled 13 x 100 test tubes. These were allowed to stand for 10 minutes before proceeding. The next step was adding 3.0 ml of reagent E with gentle vortexing giving a final volume of 4.1ml. A reagent blank was made for each batch. The blank consisted of 1.0 ml reagent D, and 3.0 ml of reagent E. The samples were allowed to develop in the dark for 30 minutes.

Following a 30 minute incubation, standards and samples were analyzed in a Beckman DU 7400 Spectrophotometer setup for a high sensitivity protein analysis at 750nm. A standard curve was made using ten known concentrations of bovine gamma globulin as shown in Tables 1, and 2.

TABLE 1. Standards for Lowry protein assay

Stock = 12.30 mg bovine gamma globulin/ 10.0 ml nanopure H_2O

Sample #	Stock	$HclO_4$	Protein concentration (final cuvette [ ])
I	500 ul	500 ul	0.15 mg
II	333 ul	667 ul	0.10 mg
III	166.7 ul	833.3 ul	0.05 mg
IV	83.34 ul	916.66 ul	0.025 mg
V	41.66 ul	958.34 ul	0.0125 mg
VI	20.82 ul	979.18 ul	0.00625 mg
VII	10.4 ul	989.6 ul	0.003125 mg

TABLE 2. Standards for Lowry protein assay

Stock = 24.6 mg bovine gamma globulin/ 10.0 ml nanopure H_2O

Sample #	Stock	$HClO_4$	protein concentration (final cuvette [ ])
A	500 ul	500 ul	0.30 mg
B	416.67 ul	583.33 ul	0.25 mg
C	333.33 ul	666.67 ul	0.20 mg

A linear regression analysis (standard concentration vs optical density) was performed on the data by the spectrophotometer. The resulting standard curve was used to quantitate the protein from the lysed cell culture samples. See figure 5. The total protein was determined for each sample. There was no pooling of samples.

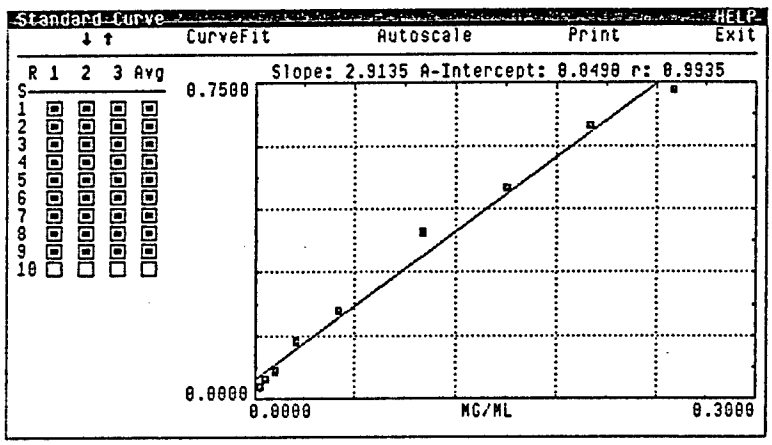


Figure 5: Lowry Standard Curve derived from standard samples I-VII and A-C.

RESULTS

Photomicrographs

Photomicrographs were taken of representative wells from each radiation and control group and cell line for the three repeated experiments. Comparison of the photos, as well as, gross observation records indicate increased cell density of the irradiated cells versus the control samples in a majority of the irradiated samples. This was independent of cell line. There were no gross morphologic differences among any of the cell lines, except the HTB-11 cell line appeared to have smaller, and more numerous cells in the irradiated wells for all energy levels; 2J, 5J, and 8J. The HTB-138 cell line appeared to have more cellular debris versus the non irradiated controls and compared to the other cell lines. Comparisons between irradiated and control wells for each cell type at 50x magnification are depicted in Plates I through XVIII. (Image scale 2.5x internal and 20x objective)

Plate I. Cellular response of ATCC HTB-11, human neuroblastoma to GaAlAs laser after 5 treatments (Tx). (A) was irradiated with 2J, (C) irradiated with 5J, (E) irradiated with 8J, (B), (D), and (F) are the non-irradiated controls for each group. Image scale x50

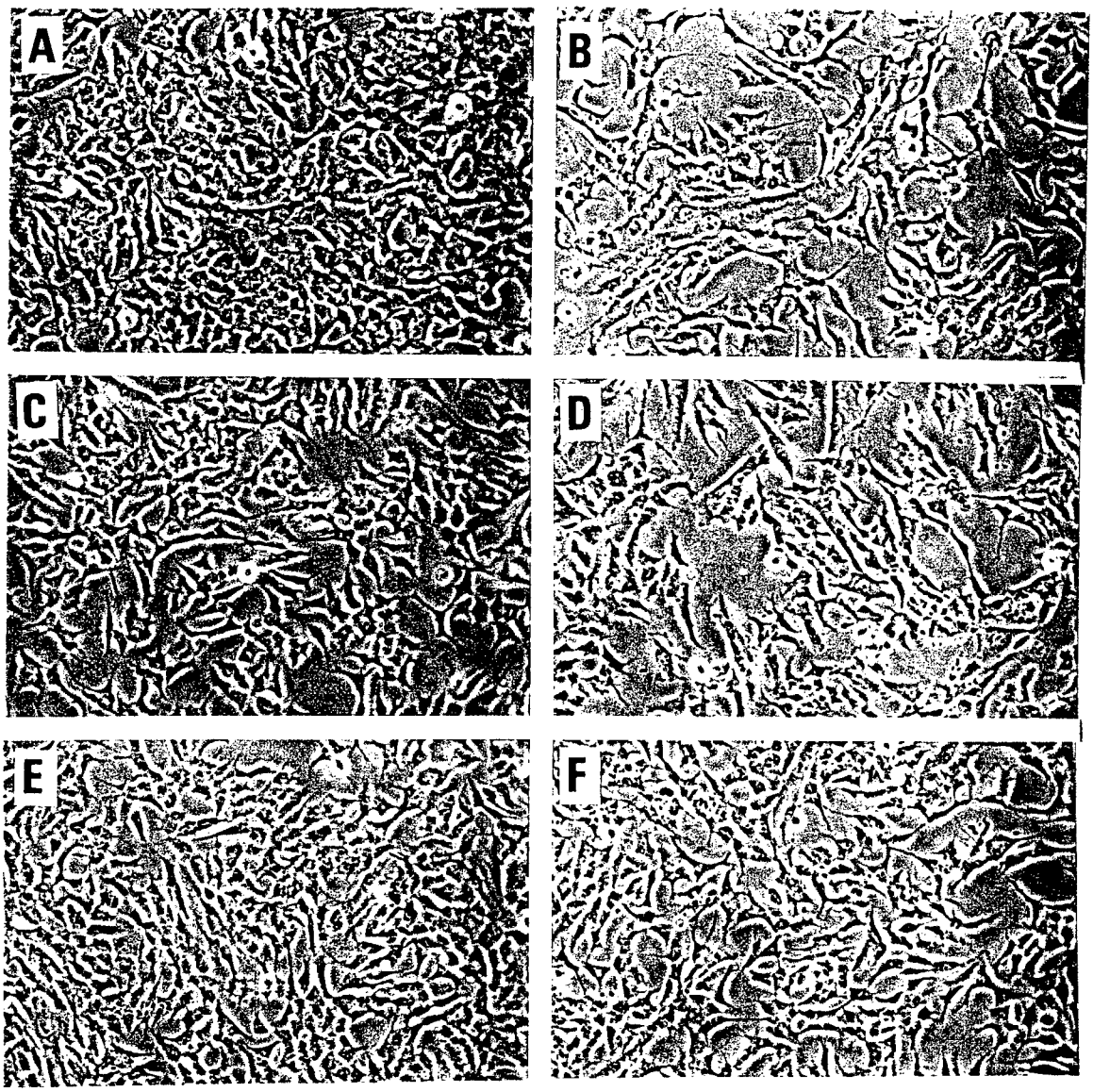


Plate II. Cellular response of ATCC HTB-11, human neuroblastoma to GaAlAs laser after 10 treatments (Tx). (A) was irradiated with 2J, (C) irradiated with 5J, (E) irradiated with 8J, (B), (D), and (F) are the non-irradiated controls for each group. Image scale x50

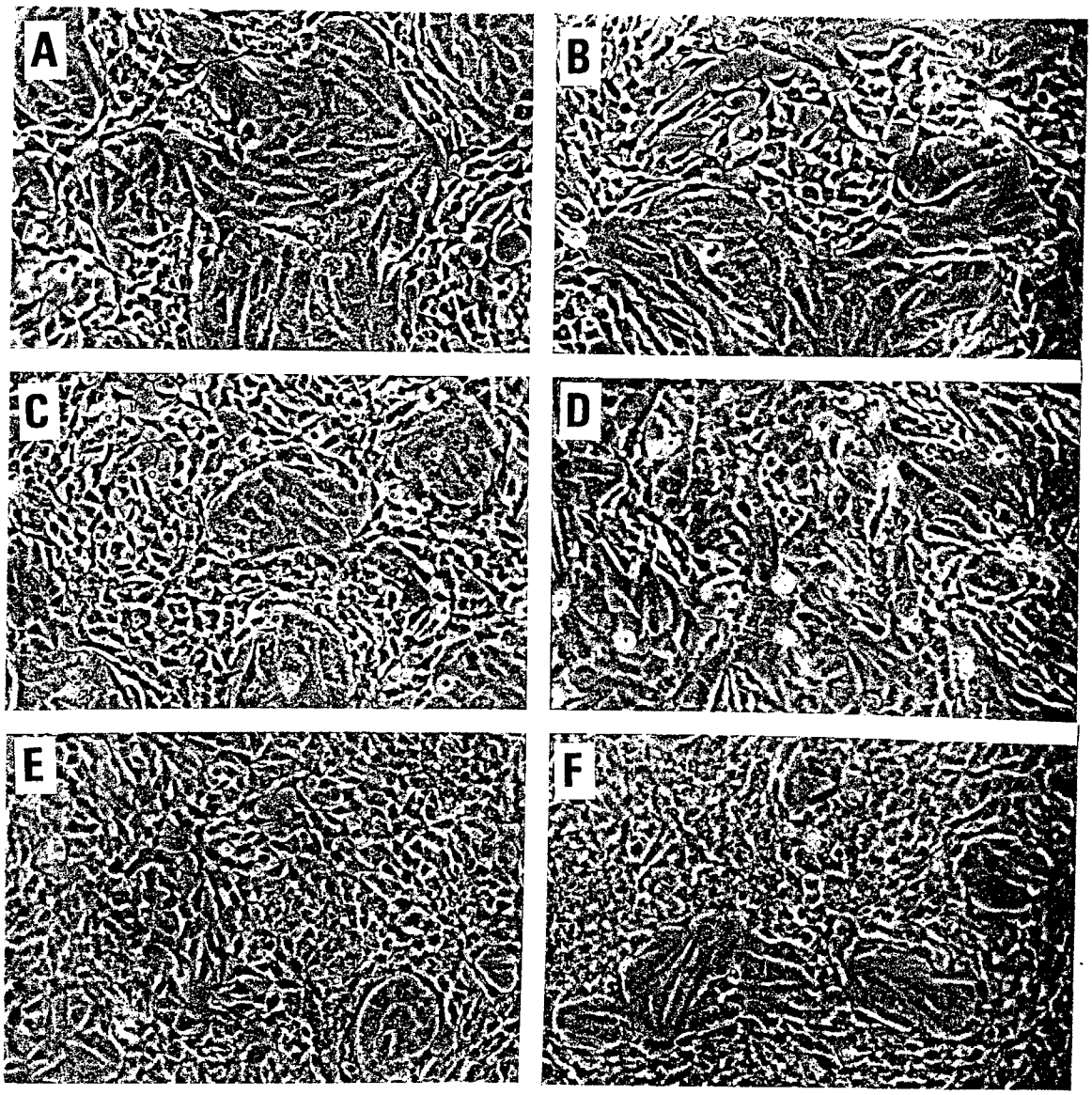


Plate III. Cellular response of ATCC HTB-16, human glioblastoma to GaAlAs laser after 5 treatments. (A) was irradiated with 2J, (C) irradiated with 5J, (E) irradiated with 8J, (B), (D), and (F) are the non-irradiated controls for each group. Image scale x50

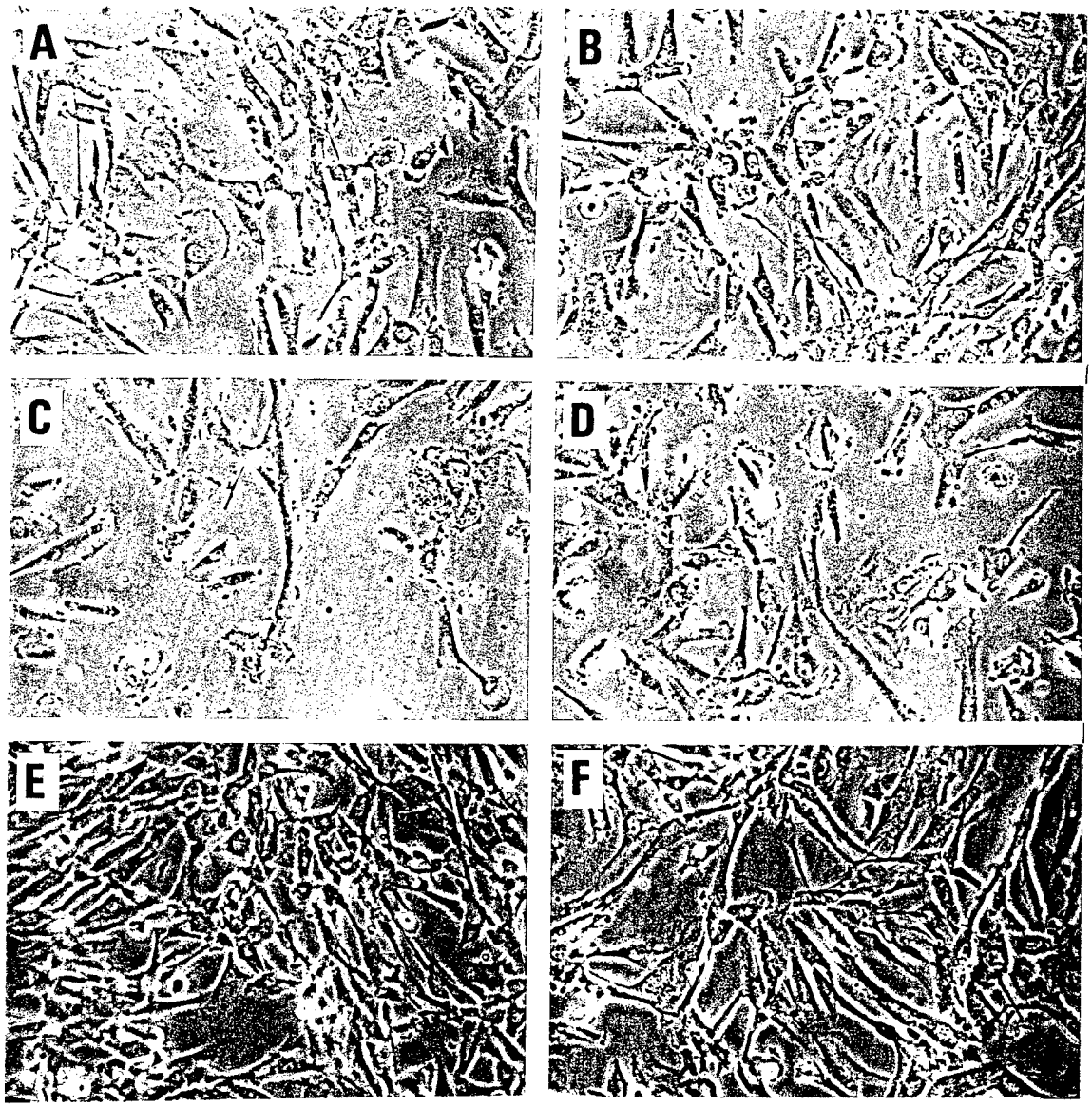


Plate IV. Cellular response of ATCC HTB-16, human glioblastoma to GaAlAs laser after 10 treatments. (A) was irradiated with 2J, (C) irradiated with 5J, (E) irradiated with 8J, (B), (D), and (F) are the non-irradiated controls for each group. Image scale x50

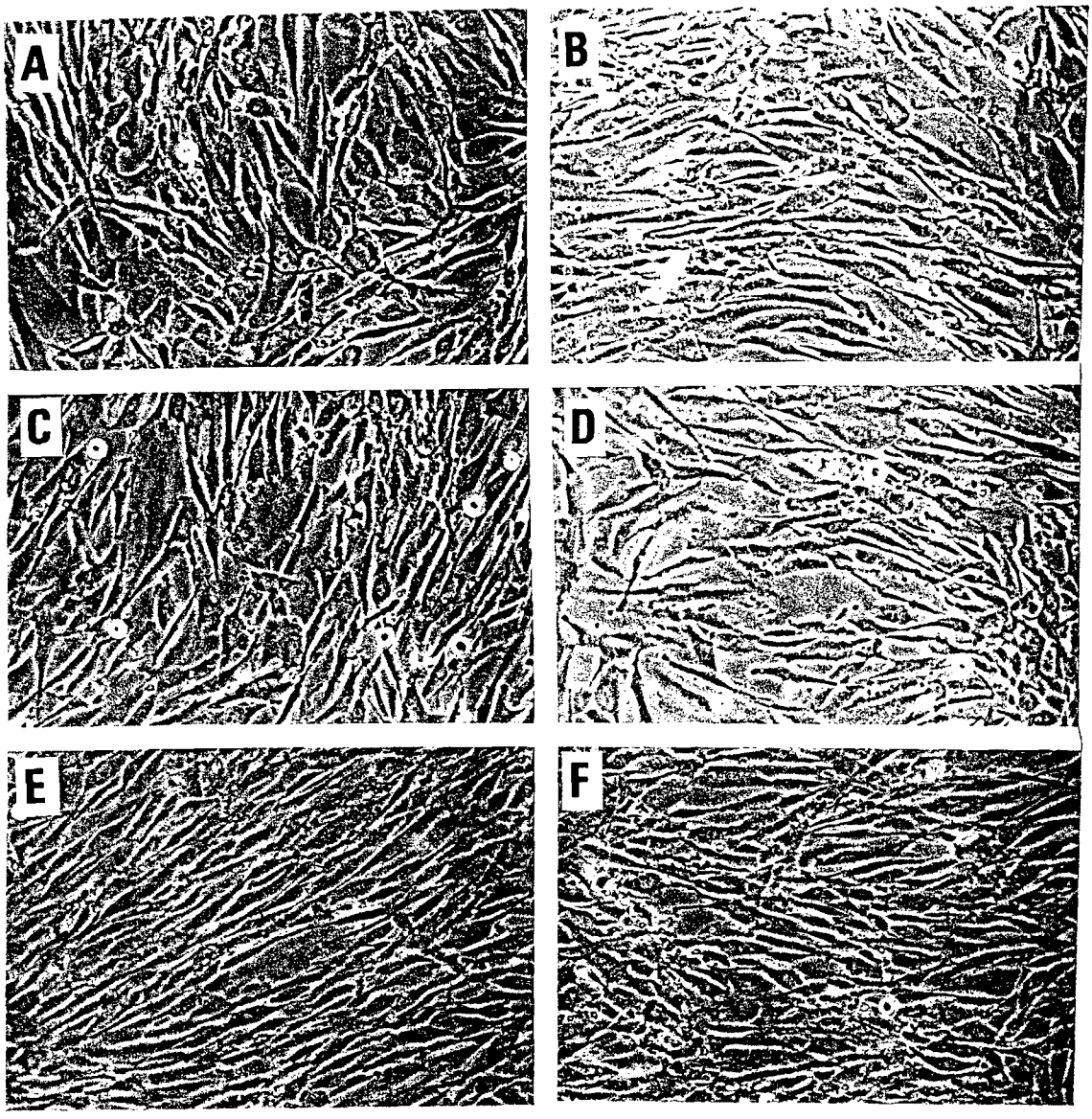


Plate V. Cellular response of ATCC HTB-138, human glioma to GaAlAs laser after 5 treatments. (A) was irradiated with 2J, (C) irradiated with 5J, (E) irradiated with 8J, (B), (D), and (F) are the non-irradiated controls for each group. Image scale x50

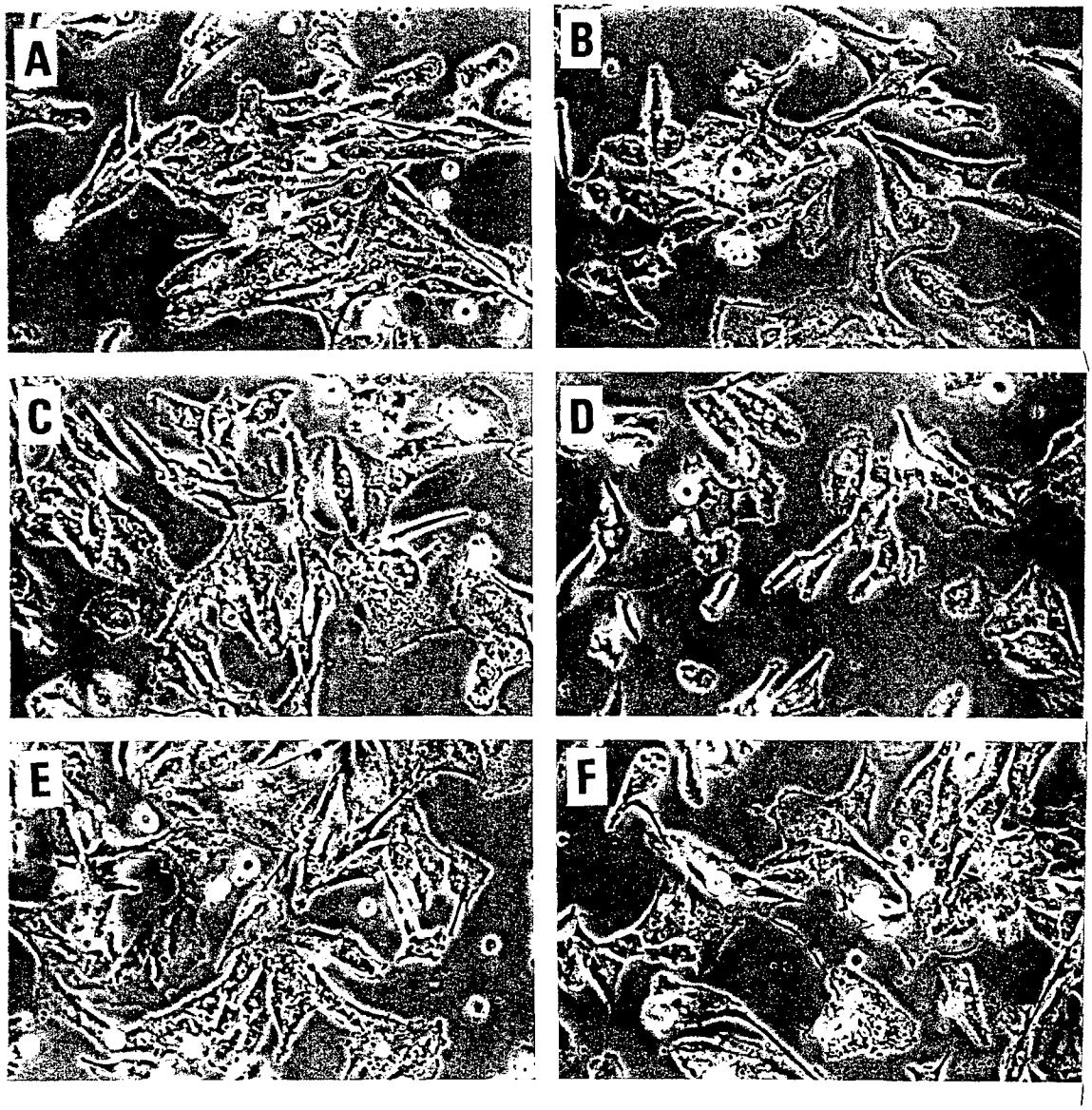
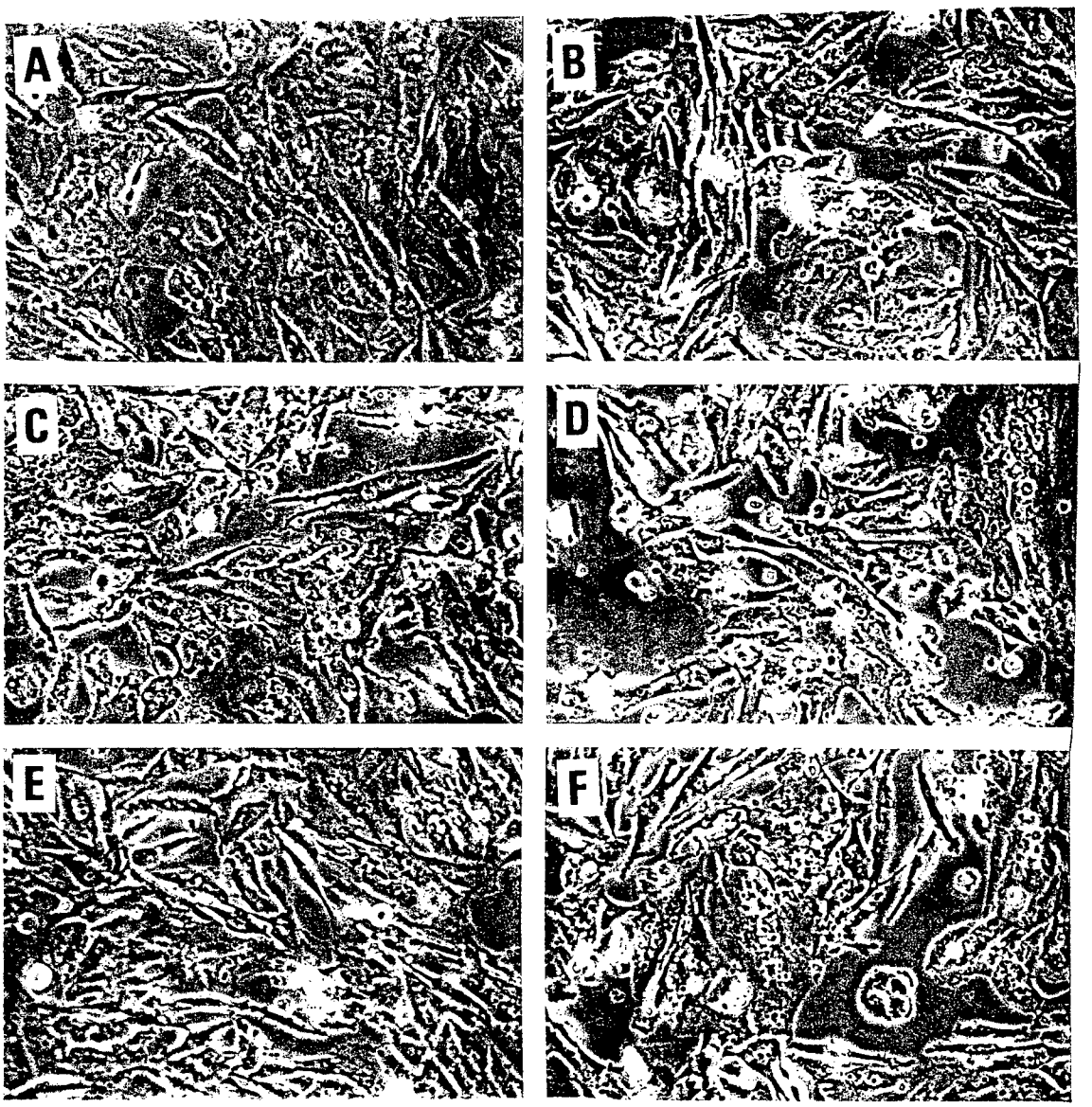


Plate VI. Cellular response of ATCC HTB-138, human glioma to GaAlAs laser after 10 treatments. (A) was irradiated with 2J, (C) irradiated with 5J, (E) irradiated with 8J, (B), (D), and (F) are the non-irradiated controls for each group. Image scale x50



Cell Count and Viability

Cell viability was measured by assessing trypan blue exclusion during cell counts. All three cell lines showed ≥ 92% cell viability for all wells harvested. There was no discernable viability difference between radiated or non-irradiated cells or number of treatments with regard to trypan blue exclusion. Cell enumeration data for each cell line is depicted in figure 6. The graph shows mean values and the ± standard deviation for all three experiments with the exception of HTB-138. The cell counts for HTB-138 is the mean of experiments 2 and 3 due to the low initial plating density in experiment 1. Due to a mathematical error this first experiment was plated at 1/2 the intended cell density. Therefore, the cell counts and total protein values for this group was thrown out. Both the individual cell counts and the graphed means show higher cell counts for all irradiated wells except at 5 treatments.

The HTB-11 and HTB-16 cell lines have relatively equal cell counts for the treated and control groups at 5 treatments for the wells irradiated with 8J. The other groups exhibited an increased cell count of the irradiated wells versus the non-irradiated wells for the cell enumeration data. The HTB-11 cell line also had larger counts at 2J compared to 5J and 8J at both 5 and 10 treatments. The HTB-16 cell line had less variation in cell counts of irradiated versus control except for the 5J, 10 treatment group. This group had the largest overall cell count difference in the treated and control groups for this cell line.

The HTB-138 line had relatively equal cell counts at 5 treatments for all three radiation doses. At ten treatments the largest difference in counts between irradiated and

control groups was seen with the 8J group, followed by the 5J group, and finally the 2J group. This is opposite to the trend noticed with the cell counts of the HTB-11, and HTB-16 cell lines.

The overall trend seen in the three cell lines is an increased cell count in all irradiated cell groups with few exceptions. The raw data was processed and an ANOVA run using SPSS software. All analyses were done within and not across cell lines. The cell count parameters compared were: the differences between the irradiated and control cell counts at each energy level; cell count differences between the different radiation doses at 5 treatments and at 10 treatments also including controls, and comparison between counts at 5 and 10 treatments including the control groups. Although trends were apparent which showed higher cell counts post irradiation, the statistical analysis revealed no significant difference between any of the experimental or control groups.

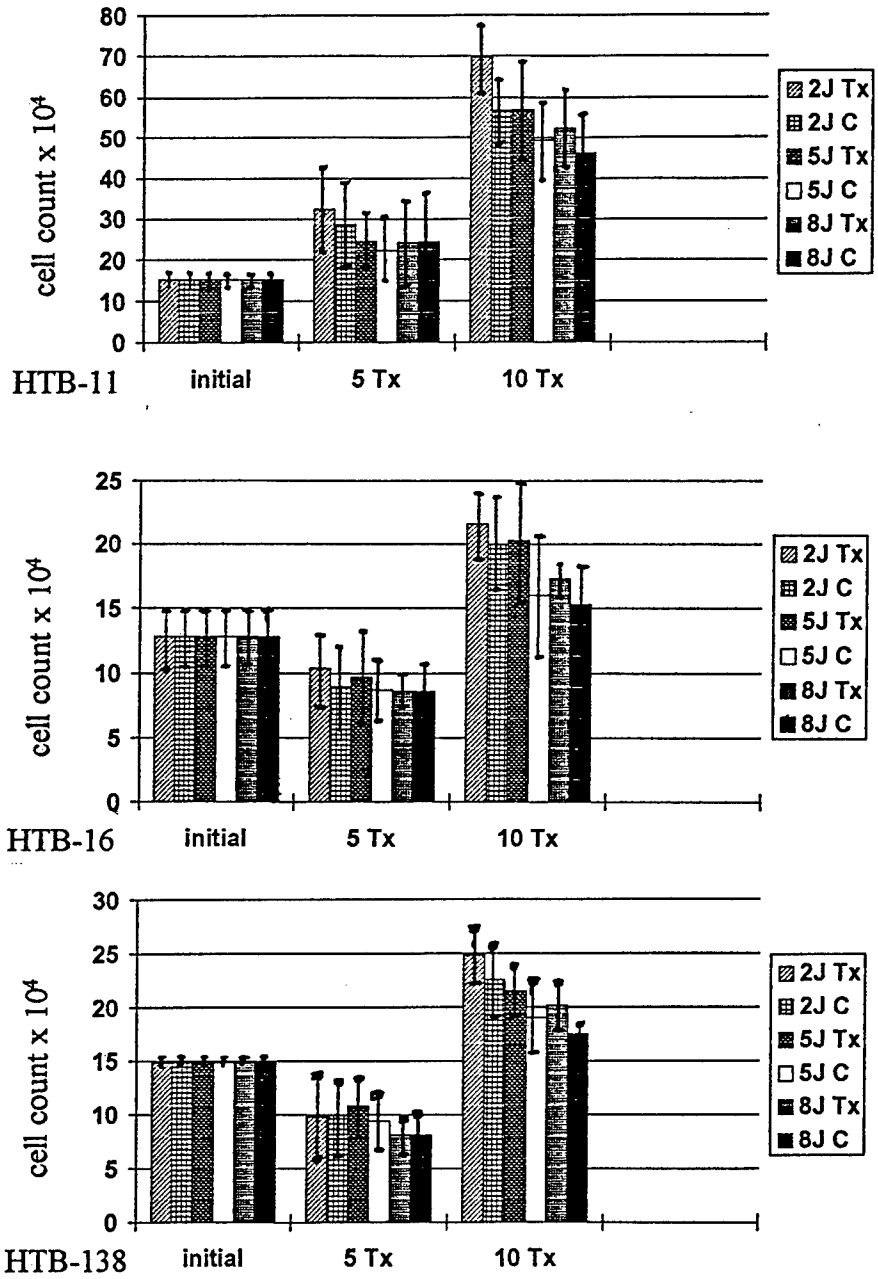


Figure 6. Mean cell count data for each cell line independently comparing initial plating density, cell counts after 5 and 10 laser treatments, and irradiation doses compared to non-irradiated controls.

Lowry Protein Analysis

The results of the Lowry total protein analysis for each cell line is seen in figure 7. These values represent the mean values for each treatment and control group with ± S.D.. The data from the HTB-138 cell line for the first experiment was omitted, again due to the low initial plating count for this cell line.

The HTB-11 cell line reveals a relatively constant total protein at 5 treatments with the exception of the 8J control and irradiated groups. These two sample groups have a lower overall concentration. At ten treatments, the 2J and 8J groups have more protein than the 5J groups. Also noted was a lower protein concentration in the irradiated vs the control groups with this number of treatments.

Protein concentrations for the HTB-16 cell line show higher values for the irradiated groups at 5 treatments, with the 2J and 5J groups being relatively equal and the 8J treated and control groups having less total protein. At ten treatments this trend reverses, with the control groups having a greater total protein than the irradiated cell samples. Comparison of the different radiation dosages shows fairly equal values for the 2J and 8J groups, with the 5J group having the lowest overall total protein.

The HTB-138 cell line exhibits fairly constant total protein values at 5 treatments for all the irradiated and control groups. At ten treatments, the 2J groups show little if any change between the values in the 5 treatment irradiated and control samples. There is an increase in total protein values at ten treatments in both the 5J and 8J groups. The 8J group has the overall highest total protein. The trend noted with the other two cell lines

is also seen here, the total protein values are higher for the control samples vs the irradiated samples at all energy levels at ten treatments.

An ANOVA using SPSS software was run on the processed values from the Lowry assay. The comparisons were the same ones performed on the cell count data, radiation level compared to control at 5 and 10 treatments, radiation levels compared at 5 or 10 treatments, with controls compared at 5 or ten treatments, and comparisons of radiation levels at 5 and 10 treatments looking for significant differences in growth rates. Comparisons were only performed within and not across the three cell lines.

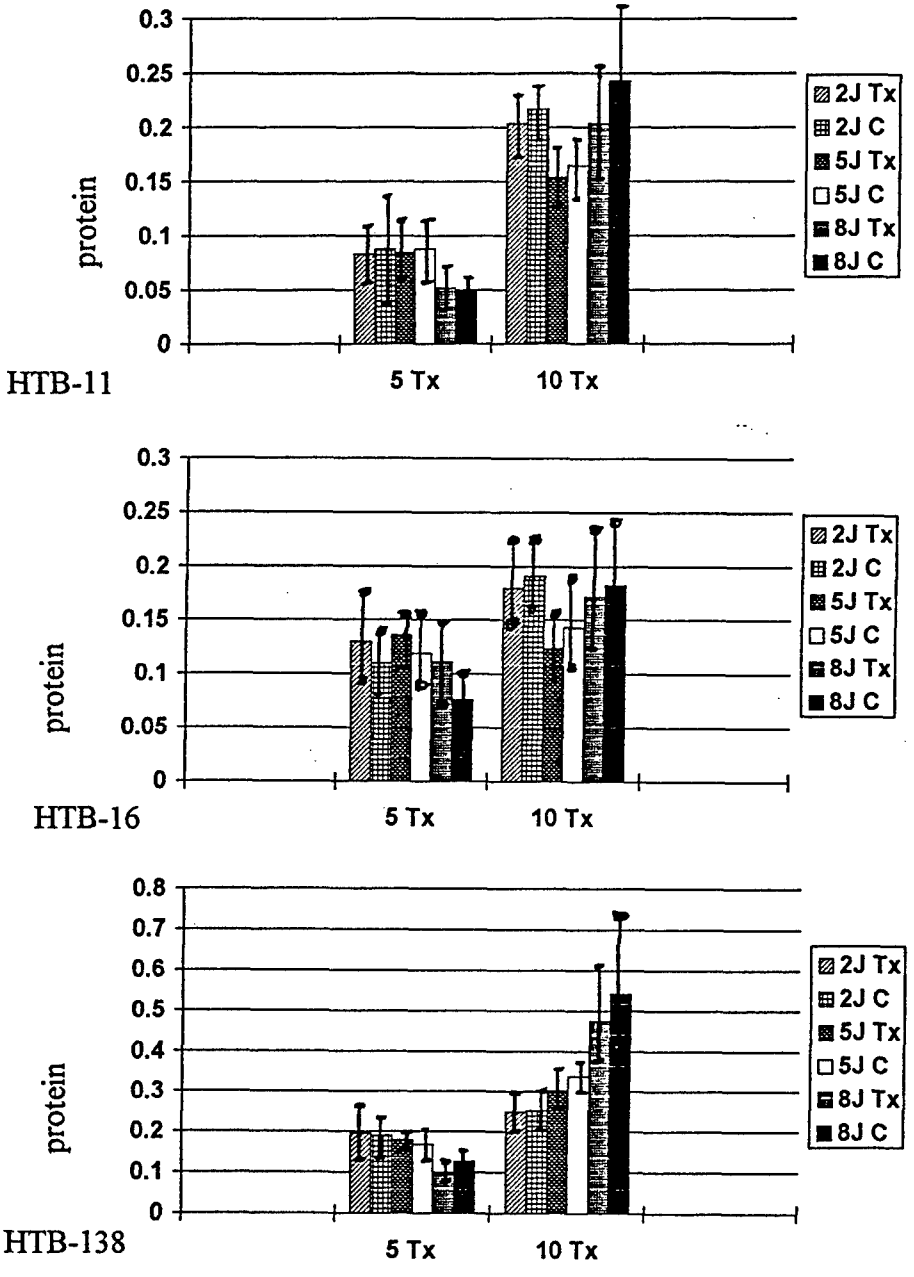


Figure 7. Mean Lowry total protein analysis data for each cell line independently. comparing values after 5 and 10 laser treatments, and irradiated cell samples compared to the non-irradiated controls.

DISCUSSION

Laser surgery is considered a treatment modality which is in the forefront of medical technology and treatment. Surgical laser therapy is relatively straight forward. A focused beam of light with a specific wavelength and a power density produces an intense heat over a confined area which can be utilized to incise, coagulate, ablate and or vaporize tissues, or any combination of these effects. However the effects of low level lasers are not quite as apparent. When reviewing the myriad of low level laser literature one may be convinced that these are the miracle therapeutic aids of the future, if not the present. Clinical data suggests that low level laser therapy augments wound healing, pain control and even nerve regeneration. Unfortunately, many of these claims have not been substantiated or repeatable.[39,40] It seems that for every experiment or trial advocating the benefits of low level lasers, there is another study claiming no significant therapeutic benefit.[23-29,35-37] One of the difficulties lies in the fact that there are many different laser systems available. In addition, there are also few controlled, prospective, and blinded studies with substantial numbers of subjects (either animal or human) reporting purely objective data. There are even fewer reports indicating appropriate treatment applications for the various uses claimed for low level lasers. Further, relatively few researchers have

looked at the cellular level to determine if there actually is a biostimulatory or even inhibitory effects of low level lasers, and the several reports published have been inconclusive.

An example of all this relative confusion can be seen in articles on nerve regeneration subsequent to crush injury in the rat model.[24-27,63] Rochkind et al. have employed this model extensively, and report favorable results of low energy HeNe lasers on sciatic nerve regeneration and repair.[24-26] With repeated doses of 6J, they noted favorable healing, decreased scar formation and enhanced tissue regeneration. Anders et al. conducted studies using the rat facial nerve and also indicated favorable results.[63] However, their delivered energy doses using a HeNe laser only demonstrated favorable results when in the 46 to 162J range. This is a considerable increase in the energy doses relative to those delivered in Rochkind's studies. A third study by Khullar and Haanaes using a GaAlAs laser at 6J energy dosing on rat sciatic nerve crush injuries indicates functional improvement vs non-irradiated control.[27] However, Khullar and Haanaes unlike Rochkind, did not detect differences in evoked potentials or any histologic differences between the irradiated and control groups, unlike Rochkind. Khullar and Haanaes concluded that the beneficial post laser treatment effects must have occurred at a site more peripheral to the area measured.

Reports of clinical trials have also shown inconsistencies. Midamba and Haanaes treated 40 patients with post surgery facial anesthesia using a GaAlAs laser and 6J treatment energy doses.[48] They report only positive subjective data, and had no control group. They concluded that low level lasers successfully regenerate peripheral nerves in

humans. A blinded clinical study by Poole, Holland and Peterson also employed a GaAlAs laser at comparable dosages with a smaller patient population.[49] Poole et al. reported subjective as well as objective data. Notably, all patients reported improvement of anesthesia, even those in the control group. Objective data revealed only one significant test out of the three tests performed.

The purpose of this current cell culture study was to test the hypothesis that low level lasers stimulate the growth and regeneration of nerve and perineural tissues in vitro. Variables were minimized and appropriate controls were incorporated in the experimental design. The qualitative observations indicate that the irradiated groups show an increased cell density, except for the 5 treatment 8J groups. This observation holds true in all three cell lines. The consistent finding at 10 treatments is increased cell counts in all the irradiated groups vs the control groups. This also holds true in all cell lines. The quantitative data, (both mean and actual counts,) show that the cell numbers increase as the irradiation dose decreases. In otherwords there are higher counts at 2J vs 5J vs 8J, in descending order. This would seem to support the hypothesis that low level lasers do indeed stimulate biological systems. Unfortunately, statistical analysis (ANOVA) does not indicate significant differences between the treated and control groups for any dose as compared within the same cell line and compared to the controls. The only significance found was between 5 treatments and 10 treatments independent of radiation or control group indicating positive growth. Notably, the laser did not inhibit cell proliferation *in vitro.*

Another interesting finding looking at the cell count charts is that the relative hardiness of the cells differed. The HTB-11 being more voracious that the HTB-16 and the least active, HTB-138. This observation can be reinforced by the ATCC product sheets. The HTB-16 and HTB-138 also had a drop in the cell counts at 5 treatments vs initial plating for both irradiated and control groups. This could also be an indicator of cell hardiness. The HTB-11 line did not exhibit a drop in cell counts at either harvest point, 5 treatments or 10 treatments. All three lines, both treated and control did show positive growth at 10 treatments compared to the initial plating density.

The other quantitative assessment, protein analysis, shows a reciprocal trend at 10 treatments relative to the cell count data, i.e., protein values were lower, albeit not significantly so in the irradiated cultures. It was noted with the HTB-11 cell line that the irradiated cells did appear more dense than the control cell groups, and they appeared to be smaller in size. This could help to explain the lower protein values in the irradiated groups as compared to the controls, i.e., the irradiated cells are replicating at a faster rate and are therefore smaller in size with less total protein than their non-irradiated counterparts. Also the Lowry assay measures intracellular protein concentration. If more protein is being exported, then there is less available intracellularly. The laser may be stimulating these cells to increase production of extracellular matrix products used outside the cell and exporting these products. There appears to be little if any correlation between the total protein data of the irradiated and control groups at 5 treatments compared to the cell count data. One of the possible reasons for this could be accuracy of any spectrophotometric protein assay. With the exception of amino acid analysis, protein

assays have approximately a 20% variation. The cell media was supplemented at the intervals indicated for optimal growth, and the cell viability determined by trypan blue exclusion at harvesting and counting indicates that the cells were healthy, 92% or greater viability for all wells.

Although our data appears contradictory, it does compare favorably to published data on *in vitro* cultured fibroblasts.[50] HeNe low level lasers were shown to increase the cell counts of human fibroblasts *in vitro* while concurrently decreasing type I collagen production. These investigators speculated that lasers stimulated specific cellular functions while suppressing others. There was no qualitative morphological assessment in this study by van Breugel et al. If the cells were indeed smaller, and replicating at a faster rate, then the metabolism should be geared more toward cellular proliferation, and not extracellular repair processes. Further, because cellular morphology has a 3 dimensional component, it is difficult to accurately determine cell size by two dimensional assessment.

Other considerations for modifications in cell growth would have to be temperature. Total irradiation time for the 8J group was greater than the 5J group, which was greater than the 2J group. Approximate total time out of the incubator for the 8J group was 50 min for 6 plates and 25 minutes for 3 plates. The time for the 5J group was 35 min and 18 min for 6 plates and 3 plates respectively, and 10 min and 5 min for the 2J group for 6 or 3 plates. This could account for subtle differences. Except for irradiation, the plates were treated on the bench or in the hood for the same amount of time.

In this group of experiments, laser dosages were 2J to 8J. These dosages correspond with the majority of reports in the literature dealing with animal models,

human trials and other *in vitro* studies. However, there is a considerable difference as to the matter through which the laser beam must pass before reaching its targeted destination. In the human and animal models, this substance is skin or mucosa and subcutaneous tissue. However, *in vitro*, the beam is transmitted exclusively through optical quality plastic and tissue culture media, thereby markedly restricting diffraction. There is an obvious difference in the densities of the substances that the laser beam is transmitted through when comparing *in vivo* and *in vitro* models. This might indicate that the radiation doses delivered in the *in vitro* models should be lowered as compared to animals and humans. Another factor is that *in vitro* models deal with a specific cell type or types exhibiting a particular heterogeneity. This would omit the effects of inter cellular interactions between widely varying tissues and organs seen in humans and animals.

SUMMARY

An *in vivo* laser effect may be the result of interactions between cell types within or adjacent to nerve tissue, or be enhanced by attenuation of laser energy by other tissues as the energy penetrates to the nerve. The present study does not support the hypothesis that there is a direct effect of the low level GaAlAs laser on neural or perineural tissue. However the trends noted suggest that an effect might be measurable with lower levels of irradiation, as may occur in the nerve *in vivo*. Further experimentation is warranted to more thoroughly evaluate the low level laser's effects and potential in tissue regeneration and repair.

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LOW LEVEL LASER IRRADIATION OF NERVE CELLS *IN VITRO*

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Low energy laser treatment of patients with nerve injuries has been reported to achieve enhanced return of sensation in treated patients. Animal studies have shown reduction in scar formation and improved function following laser treatment of crushed sciatic nerves. However, these results remain controversial. Other clinical and animal studies fail to find any laser effect, and the biological basis for an effect has not been established. Studies of cultured fibroblasts have produced conflicting results, and there is little *in vitro* data regarding laser effects on nerve tissue. The purpose of this study was to determine the effects of GaAlAs low energy laser irradiation of rat cerebral cortical cells, and human nerve cells *in vitro*.

Primary rat cerebral cortical cells were obtained for the first group of three experiments. Numerous problems were encountered with growing these cells, so the protocol was modified to use established human cell lines. Human neuroblastoma, glioblastoma, and glioma cell culture lines (American Type Culture Collection, Rockville, MD) were each plated at uniform density in paired wells in multiple six-well plates. One well per plate of each cell type was then irradiated with 2, 5, or 8 Joules per day for either five or ten days, using a

70 mW GaAlAs laser system (Ronvig Instruments, Denmark). Each experimental group consisted of a total of 9 wells, each having an adjacent untreated control well. After treatment was complete cells were photographed, then harvested and counted. A Lowry protein assay was performed on all harvested cell groups. The results were analyzed using an SPSS ANOVA analysis.

Gross microscopic observation revealed denser-appearing cultures in the irradiated groups with few exceptions. In all groups except one, mean cell counts were higher in the irradiated groups than in the paired controls. Cell counts were higher in groups treated with lower daily energy doses than in those which received higher doses (2 J > 5 J > 8 J). Protein analysis revealed a lower total protein in the irradiated versus the control groups. However, the experiments do not demonstrate statistically significant differences in cell counts or total protein between laser-treated groups and controls or among different laser-treated groups.

An *in vivo* laser effect may be the result of interactions between cell types within or adjacent to nerve tissue, or be enhanced by attenuation of laser energy by other tissues as the energy penetrates to the nerve. The present study does not support the hypothesis that there is a direct effect of the low level laser energy on perineural or neural tissue. However, the trends noted suggest that an effect might be measurable with lower levels of irradiation, as may occur at the nerve *in vivo*, and that further experimentation is warranted.